HYALURONIDASE: PURIFICATION AND INHIBITION BY A GOLD COMPLEX AND A STEROID DERIVATIVE

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Gary Gerard Durante

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VITA

Gary G. Durante, Lieutenant U.S. Navy, son of Paul and Diane (Zakrewski), was born March 14, 1958, in New Hartford, New York. He attended Holy Trinity grammar school and graduated from Notre Dame High School, New Hartford, New York, in 1976. In August, 1976, he entered the Citadel, The Military College of South Carolina, and was awarded the degree of Bachelor of Science (Biology) and commissioned an Ensign in the U. S. Navy on May 17, 1980. In July, 1980, he commenced training to obtain qualifications for supervision of gas turbine propulsion plants and graduated from the Surface Warfare Officer School, Newport, Rhode Island, in November, 1980. He served one sea tour from December, 1980, until July, 1983, on the gas turbine powered destroyer USS O'Bannon (DD-987). From July, 1983, to August, 1985, he served a tour of duty as an instructor at the U. S. Navy Gas Turbine Engineering School in Newport, Rhode Island. In September, 1985, he commenced graduate studies under the Navy's Post Graduate Education Program. He married Pamela Marie, daughter of Thomas and Leota Fisher, in July, 1984.

THESIS ABSTRACT

HYALURONIDASE: PURIFICATION AND INHIBITION BY A GOLD COMPLEX AND A STEROID DERIVATIVE

Gary Gerard Durante

Master of Science, December 11, 1987
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The enzyme hyaluronidase from human synovial fluid was partially purified using gel-filtration and chromotography on a hydroxylapatite column. A 4.1-fold purification of hyaluronidase having a specific activity of 1.1×10^{-4} μmol/min/mg was obtained. The enzyme was inhibited by the gold complex Auranofin, and the steroid derivative, 22-ketocholesterol oxime.

It was shown that the complexes differed in their ability to inhibit the enzyme. The rate constants and equilibrium inhibition constants were calculated. The K_I value for Auranofin was 8.78 mM and its rate constant was 0.049 min^{-1}. The K_I value for 22-ketocholesterol oxime was 11.86 mM. Kinetic analyses demonstrated an apparent uncompetitive inhibition by the steroid derivative 22-ketocholesterol oxime.

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LIST OF ABBREVIATIONS

A	absorbance
Auranofin	S-2,3,4,6-tetraacetyl-1-ß-D-thioglucose (triethylphosphine) gold (I)
BSA	Bovine Serum Albumin
cm	centimeter
EDTA	ethylenediaminetetraacetate
gm	gram
h	hour
Km	Michaelis constant
l	liter
ma	milliamperes
mg	milligram
min	minute
ml	milliliter
mM	millimolar
nm	nanometer
t	time
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
v	velocity
v/v	volume per volume
µM	micromolar
µg	microgram

μl	microliter
K_I	inhibition constant
k_2	rate constant
Vmax	maximum velocity

I. INTRODUCTION

Arthritis and rheumatism are frequently used words which are often employed without a clear understanding as to their underlying meaning or knowledge of their causes.[1] Arthritis is the general term used when the joint itself is the major site of the rheumatic disease, while rheumatology is the study of the rheumatic diseases, including arthritis, fibrositis, rheumatic fever, bursitis, neuralgia, gout, and other conditions producing stiffness, soreness, or somatic pain.[2] According to the estimates obtained from numerous surveys, and quoted by the Arthritis Foundation in 1982,[3] there are more than 36,000,000 persons in the United States suffering from some sort of arthritis or related disease.

Rheumatoid arthritis is one of the most prevalent types of rheumatic disease and has received increasing interest by various disciplines within the scientific community. It is relatively common, and in its more serious forms it can lead to severe crippling and disablity. The origin of rheumatoid arthritis has eluded detection despite a great expenditure of resources and energy. It appears that the pathology of rheumatoid arthritis is undoubtedly related to an

inflammatory response involving the immune system, and the initiating event which triggers this response is most likely a specific etiologic agent.[2]

Recently, the interest of most workers have focused on immunologic phenomena as the instigators of the primary pathogenetic mechanisms in the causality of rheumatoid arthritis.[4-6] The most common way to account for inflammation in a rheumatoid joint, in the absence of any known etiologic agent, is to invoke an altered immune reaction. There is evidence to suggest that immunologic events occur in rheumatoid arthritis, particularly in the joints. Rheumatoid factors have been observed with rheumatoid arthritis. Immune complexes formed between rheumatoid factors and γ globulin are phagocytized by leukocytes in vitro[7] or in the synovial fluid in the joint.[8] As a result of phagocytosis of particulate complexes, leukocytes may degranulate[9] releasing their lysosomal and hydrolytic enzymes into the joint fluid, possibly provoking local inflammation.

Analysis of synovial fluid plays a major role in the diagnosis of joint disease. Synovial fluid is basically an ultrafiltrate of the plasma combined with a mucopolysaccharide (hyaluronate) that is synthesized by the cells of the synovial membrane.[10] The functions of the synovial fluid are to lubricate the joint space and transport nutrients to the articular cartilage.[11]

Destruction of the synovial fluid with age or in a disease state may lead to the development of degenerative joint disease (osteoarthritis). Inflammatory joint fluids contain lytic enzymes that produce depolymerization of hyaluronic acid greatly impairing the lubricating ability of the fluid.[11]

Every year additional enzymes have been found in elevated concentrations in rheumatoid joint effusions leading to an already impressive list. Examples illustrating the diversity of these enzymes are given in Table 1.

There is indirect evidence to support the idea that the enzymes are not circulatory since their activities in the sera of rheumatoid patients are found to be within normal limits.[25,26] It has been shown in inflammatory diseases that synovial cells,[27] leukocytes infiltrating the synovial tissue,[18] and cartilage cells[28] are all involved in the increase of the afforementioned enzymes in synovial fluid.

Table 1. Enzymes in Synovial Fluid of Patients with Rheumatoid Arthritis.

Enzyme		Reference
Lactate dehydrogenase	(EC 1.1.1.27)	12
Pyruvate kinase	(EC 2.7.1.40)	12
Aspartate aminotransferase	(EC 2.6.1.1)	12
5'-Nucleotidase	(EC 3.1.3.5)	13
Leucine aminopeptidase	(EC 3.4.11.1)	13,14
Pepsin	(EC 3.4.23.1)	15
Trypsin	(EC 3.4.21.4)	15
Acid phosphatase	(EC 3.1.3.2)	16
Alkaline phosphatase	(EC 3.1.3.1)	17
Cathepsin D	(EC 3.4.23.5)	18
Cathepsin G	(EC 3.4.21.20)	19
Lysozyme	(EC 3.2.1.17)	19
α- Mannosidase	(EC 3.2.1.24)	20
N-Acetyl-β-D-glucosaminidase	(EC 3.2.1.30)	21,22
β-Glucuronidase	(EC 3.2.1.31)	23
Myeloperoxidase	(EC 1.11.1.7)	19
Collagenase	(EC 3.4.24.3)	24

Swann[29] classified, on the basis of their origin, the synovial fluid macromolecular constituents as follows: a) the constituents derived from blood ; b) the substances secreted by the joint tissues; and c) the products that were derived from the catabolism of the joint. Enzymes and regulators of enzymatic activity have been shown to originate from each of these sources. While it is probable that many of the enzymes that are featured predominantly in diseased joints are derived from infiltrating leukocytes,

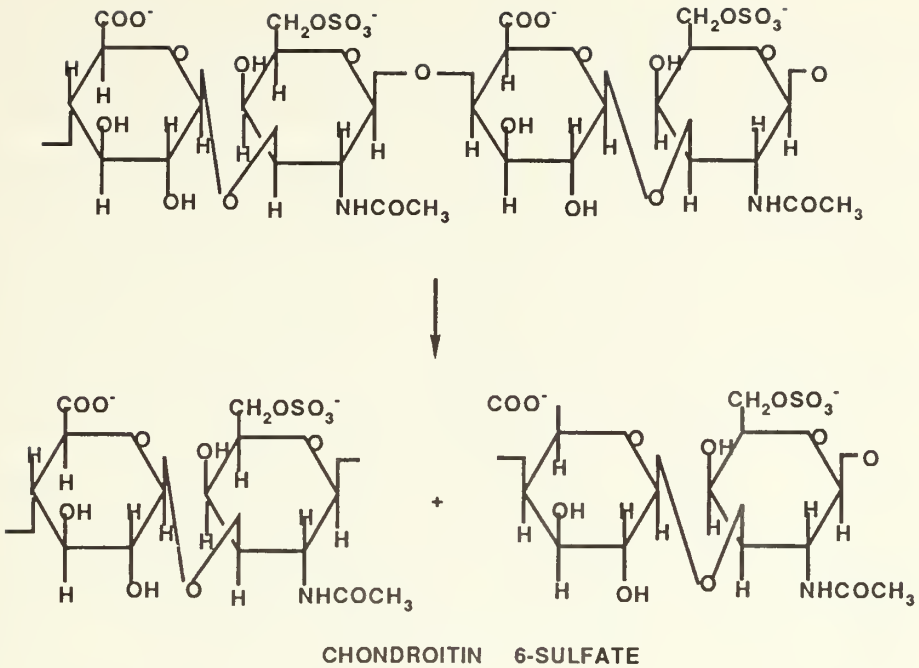
recent work has made it clear that in some cases they are derived from the non-vascularized intra-articular tissues such as synovium and cartilage.[18]

Hyaluronidase, discovered by Duran-Reynals,[30] constitutes a family of enzymes. They can be grouped into various types according to their mechanism of action.[31] Hyaluronoglucosaminidases (EC 3.2.1.35), hyaluronoglucuronidases (EC 3.2.1.36) and hyaluronate lyases (EC 4.2.2.1) comprise several isoenzymes.[32] For example, testicular and lysosomal hyaluronidases are both of the aminidase type, but the former has an optimum pH of 6.0 and the latter a pH of 3.5.[33]

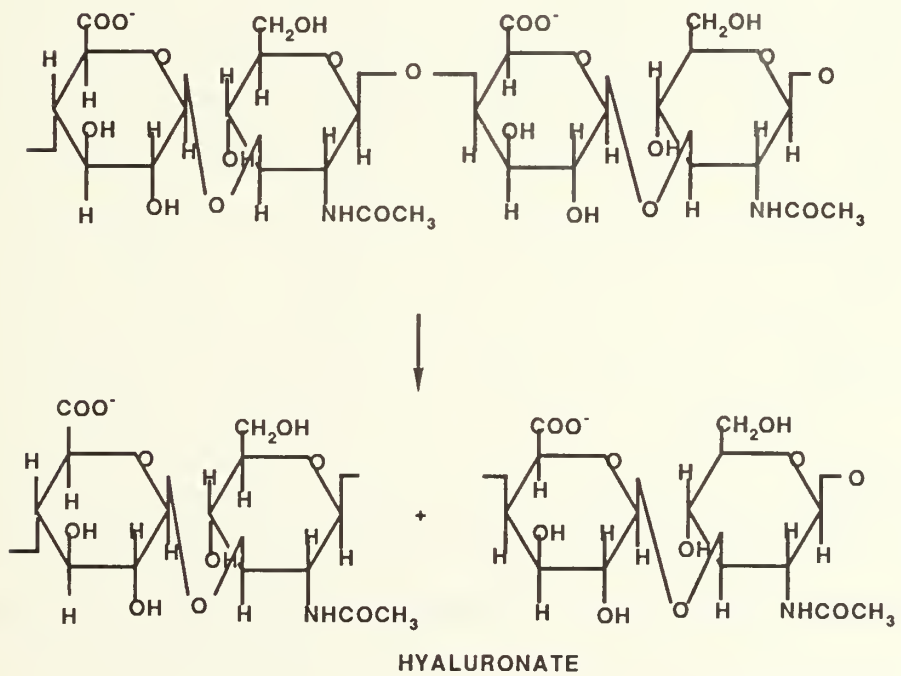
Hyaluronidases can also be separated into two groups according to their physiological role. They can function in the metabolism of "self" hyaluronic acid, and yet act upon "non-self" substrates. The first group of hyaluronidases comprise the lysosomal and serum hyaluronidases which are characterized by an in vitro narrow acidic pH range of activity. The second group, the testicular, bacterial, and venom hyaluronidases, are enzymes with an in vitro broad pH range of activity whose optimal pH is close to neutrality.[34]

Hyaluronidases hydrolyse the (β 1-4) glycosidic bonds in a number of different substrates such as hyaluronic acid, chrondroitin 4-sulfate and chrondroitin 6-sulfate.[35] (Figure 1) These substances are essential for maintaining

the integrity of the extracellular fluid matrix. Alteration of their structure and concentration may affect and modulate numerous cell functions, such as, phagocytosis,[36,37] adhesion,[38] mobility,[39] proliferation,[40,41] and differentiation.[42,43] The hyaluronic acid-hyaluronidase system takes part in the process of dermis permeability,[44] embryonic development,[45] tumor development,[46,47] cellular immune response,[48,49] and in the control of the angiogenisis factor.[50] It also plays an important role in such processes as fertilization,[51-53] bacterial infection,[54-58] and venom dispersion.[59-62]



CHONDROITIN 6-SULFATE



HYALURONATE

Figure 1. Action of Hyaluronidase on Different Substrates.

Hyaluronidase in the present study was obtained from human synovial fluid and canine synovial fluid. The properties of this enzyme from different sources have been thoroughly investigated by Meyer, Hoffman and Linker.[63] It has been shown that hyaluronidase from rat liver[64,65] and from fetal rat bone [66] is localized in the lysosomes of those tissues. The liver enzyme was shown to give an even numbered of oligosaccharide products, which are identical to those produced by the testicular enzyme.[64] Aronson and Davidson[67] isolated hylauronidase from the lysosomes of rat liver. The enzyme was purified 1300 fold by ammonium sulfate precipitation, chromotography on hydroxyapaptite and chromotography on Sephadex G-100. The purified fraction gave two peaks, the slower of which yielded a single band using acrylamide gel electrophoresis while the faster of the two was yellow and contained no enzymatic activity. The molecular weight of the slower peak enzyme was calculated to be 89,000 based on sedimentation equilibrium data.[67]

Joy et al[35] investigated liver hyaluronidase using pigs as their source of the enzyme. Their technique of purification varied from that of Aronson.[67] A single band was observed after electrophoresis on polyacrylamide gel yielding an enzyme with an apparent molecular weight of 70,000 and an isoelectric point of 5.0. The substrate specificity of this enzyme was the same as that for bovine testicular hyaluronidase ; however, the Km and Vmax values

of the porcine liver enzyme were significantly lower for all substrates (hyaluronate, chrondroitin 4-sulfate, chrondroitin 6-sulfate).[35] The amino acid composition of hyaluronidase isolated from bee venom by Kenemy[69] is illustrated in Table 2.

Table 2. The Amino Acid Composition of Purified Bee Venom Hyaluronidase

Amino acid	Amount in hyaluronidase found unoxidised(a)	oxidised(a)	Reported (b) unoxidised
Aspartic acid	15.6	16.6	15.9
Threonine	7.0	5.6	6.9
Serine	6.7	6.6	8.6
Glutamic acid	16.2	15.4	11.9
Proline	10.0	9.7	5.3
Glycine	10.1	9.7	8.2
Alanine	9.4	9.4	7.1
Valine	4.2	4.1	6.1
Methionine	0.6	----	1.96
Methionine sulfone	----	1.3	----
Isoleucine	2.8	2.9	4.0
Leucine	10.0	10.0	10.0
Tyrosine	5.7	----	6.5
Phenylalanine	5.7	----	5.0
Lysine	6.9	7.1	9.1
Histidine	1.3	2.0	2.8
Arginine	8.7	8.8	7.8
Half-cysteine	----	----	1.89
Cysteic acid	----	1.1	----

The results obtained on an LKB 4400 automatic amino acid analyser are expressed as a molar ratio, taking leucine as 10.
(a) Results reported by Kemeny.[68]
(b) Results reported by King.[69]

The precise role and specificity of hyaluronidase from different sources has yet to be demonstrated. Stephens et al,[70] compared the properties of human synovial fluid and serum hyaluronidase in an attempt to elucidate the origin of this enzyme and explain the lack of correlation of the enzyme activity with inflammatory indices. Bovine testicular hyaluronidase which has been used by many workers as a model system for examining the effects of drugs and other agents on human hyaluronidase has also been investigated for its properties.[71-73]

Purification of the human synovial enzyme was acomplished by ammonium sulfate fractionation, agarose gel permeation chromatography and hydroxylapatite gel chromotography. Serum and synovial fluid enzymes gave essentially the same pH-activity relationship and behaved as typical lysosomes. Their pH optimum was approximately 3.4 with no activity produced above pH 5.0 while bovine testicular hyaluronidase had optimal activity at pH 5.0 and very low activity at pH 3.4.[70]

According to Stephens et al,[74] in contrast to the findings dealing with exopolysaccharides, the hyaluronidase activity in fluids obtained from the pathological groups consisiting of rheumatoid and osteoarthritic patients were found to be similar. The hyaluronidase activity levels of both the rheumatic and osteoarthritic samples were always less than that for normal serum. Hyaluronidase in synovial

fluid does not appear to be a product of inflammatory cells within the joint. In view of its low molecular weight estimated to be 60,000 by column chromotography, it is likely that the enzyme may be derived from a remote source such as diffusion from serum.[74] Serum hyaluronidase is indeed very similar[75] in properties to the synovial fluid enzyme and is known to be inhibited in the same manner as the serum enzyme by a substrate universally present in mammalian blood.[76]

This result, coupled with the requirements for acidic conditions makes it unlikely that the 3-enzyme system (Figure 2)[74] is active in synovial fluid in vivo. Rather, the lysosomal environment within phagocytic cells would appear as the most favorable one for glycosaminoglycan catabolism by the mechanism outlined.[74]

Different types of drugs have been used in controlling the symptoms of rheumatoid arthritis with no one drug leading to a complete cure. There is a wide and unpredictable variation of patient response to the different drugs. At the present time, non-steroidal anti-inflammatory drugs and slow acting anti-rheumatic drugs are being used to treat the disease.

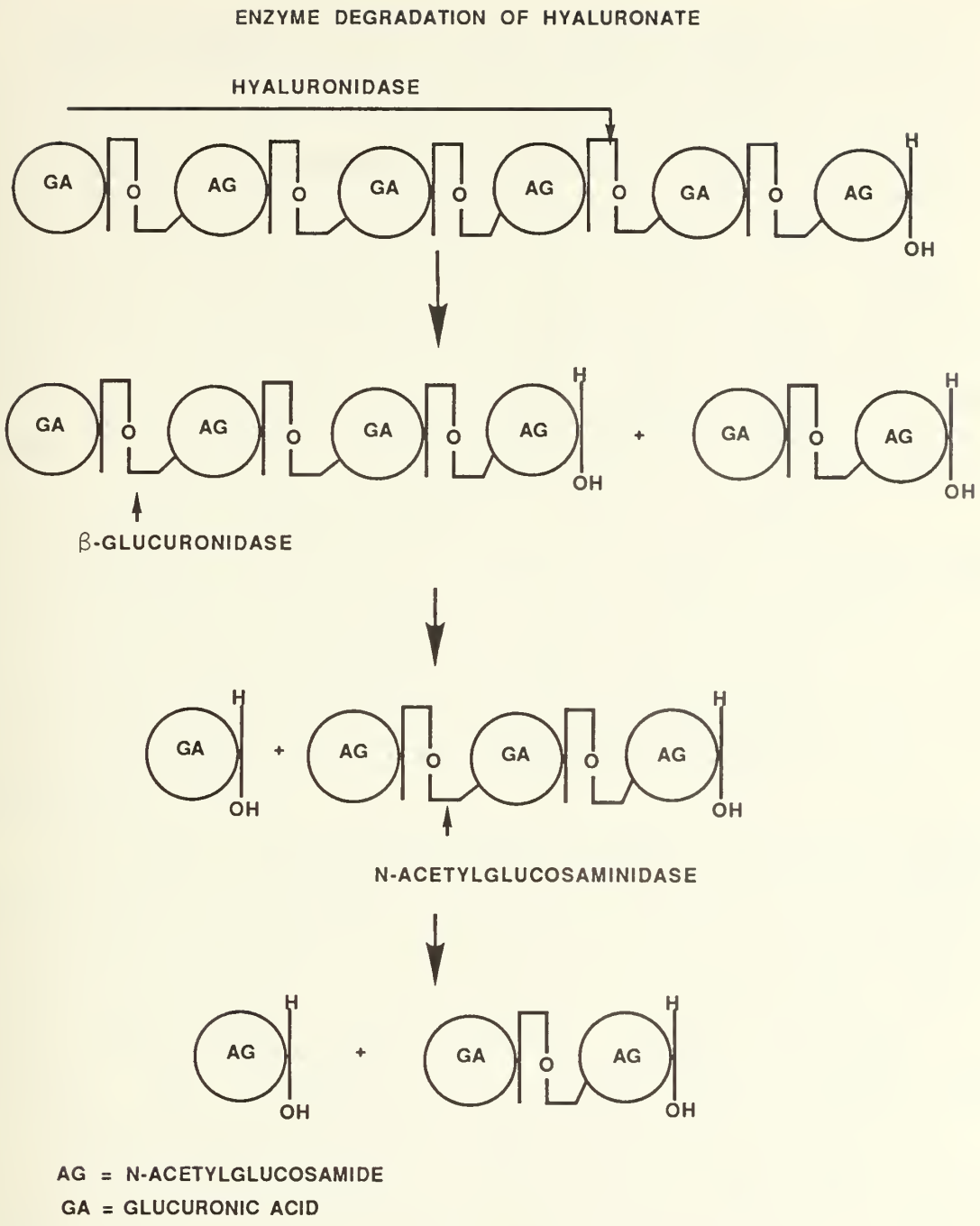


Figure 2. Scheme for the Mechanism of Total Degradation of Hyaluronate by Rheumatoid Synovial Fluid. High molecular weight substrate is first cleaved by hyaluronidase, and the oligosaccharides produced are further attacked by exopolysaccharidases.

Among rheumatologists in the United States, gold is currently the most popular long-term suppressive drug for the treatment of rheumatoid arthritis,[77] and it has been used successfully for the past sixty years. Gold is available in parenteral as well as oral form.[78] During parenteral administration, gold binds to albumin, and then tends to concentrate in the liver, kidney, spleen and lymph nodes.[79] Gold also concentrates in synovial tissues but not in cartilage. Autoradiography has shown increased concentrations of gold in subsynovial membranes in the area of perivascular infiltration of mononuclear cells.[80] Myochrysine (gold sodium thiomalate) and Solganal (aurothioglucose) are the preparations of choice for parenteral use.[77]

The exact mechanism of gold action is unknown. Besides antibacterial properties, gold exhibits partial suppressive phagocytic activity on macrophages and neutrophils. Gold stabilizes lysosomal membranes, thus inhibiting enzyme release. Most investigators, in recent years, suggest both an immunostimulative and an immunosuppressive effect of gold; thus, it may be an immunoregulating agent.[81] Biggs et al[82] recently demonstrated that all parenteral gold compounds, that is, gold sodium thiomalate, aurothioglucose, and sodium aurothiosulfate, (Figure 3) bind to the serum proteins at the same rate and exhibit similar affinity constants and labelling capabilities. The mechanism of this

reaction is not well understood, but it is likely to involve the well-recognized property of the gold compounds' ability to interact avidly with the sulfhydryl side chains of proteins.[83,84] Lorber et al[85] reported that gold shows a concentration-dependent affinity for other serum compounds including immunoglobulins and complement. Finally, injectable gold shows affinity for other circulating elements of the blood including erythrocytes[86] and lymphocytes.[87]

Auranofin is a synthetic gold compound in which the central atom of gold is stabilized by covalent bonding with two side groups, a phosphine ligand and a sulfur ligand.[88] Unlike parenteral gold, Auranofin exhibits cellular-binding rather than almost exclusively binding to serum proteins. From recent studies, it was demonstrated that 40% to 66% of intramuscualar gold is associated with cells,[89-91] primarily erythrocytes following oral administration. The balance was found to be largely bound to albumin. Cell specific binding might possibly represent part of a biologic reservoir or transport system as discussed by Stephens.[67]

Auranofin has proven to be quite effective in reducing carrageenan-induced edema in a model of inflammation as shown in the studies by Walz et al.[92] Inhibition of edema formation of 62% to 80% were obtained with varying doses of the drug. In contrast, gold sodium thiomalate and gold

sodium thioglucose did not significantly inhibit the rat paw formation edema.

Corticosteroids have played a prominent role in the therapy of rheumatic diseases since their discovery in the late 1940s. The various metabolic, anti-inflammatory, and immunosuppressive effects of the corticosteroids are the result of a common mode of action on the cells involved in rheumatoid inflammation.[93] Some steroids are known to inhibit the production of proteinases such as collagenases and plasminogen activator by synovial cells and macrophages.[94-96]

In an active site-directed mechanism, the enzyme binds with the inhibitor in forming a non-covalent complex prior to forming a covalent enzyme-inhibitor complex.[97] Kitz and Wilson[98] derived an equation that is based on the appropriate assumption that the enzyme is a reagent, as contrasted to the less rigorous assumption of steady state.[99] In affinity labeling,

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E^*I \xrightarrow{k_3} E\text{-}X \qquad (1)$$

$$K_I = k_2 / k_1 \qquad (2)$$

it is assumed that formation of the reversible complex (E * I) is in rapid equilibrium compared to the formatiom of the irreversibly inactivated enzyme (E * X) that (I) >> (E);

and that when the reaction mixture is assayed for enzymatic activity, E and E * I produce full activity while E - X is inactive. Then

$$d\,[E\text{-}X] / dt = k_3\,[E^*I] \qquad (3)$$

$$K_I = [E]\,[I] / [E^*I] \qquad (4)$$

$$[E]_T = [E] + [E^*I] + [E\text{-}X] \qquad (5)$$

$$[E]_T = [E^*I]\,K_I / [I] + [E^*I] + [E\text{-}X] \qquad (6)$$

$$d[E\text{-}X] / dt = k_3\,([E]_T - [E\text{-}X] / (1 + K_I/[I]) \qquad (7)$$

$$d[E\text{-}X] / [E]_T - [E\text{-}X] = k_3\,dt / (1 + K_I/[I]) \qquad (8)$$

Integrating,

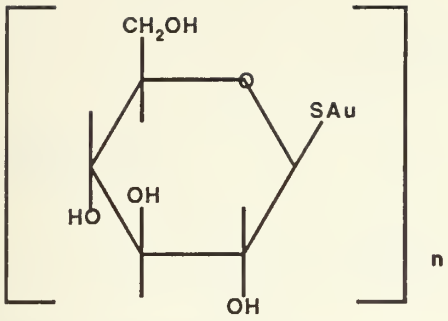
$$\ln([E]_T - [E\text{-}X]) = -k_3 t/(1 + K_I/[I]) + \ln [E]_T \qquad (9)$$

If the logarithim of enzymatic activity $[E]_T - [E\text{-}X] = [E] + [E^*I]$ is plotted against time, the observed first order rate constants, k_{obs}, may be calculated from the slope or by $k_{obs} = 0.693/t_{1/2}$ and is related to the desired constants by equation 10.

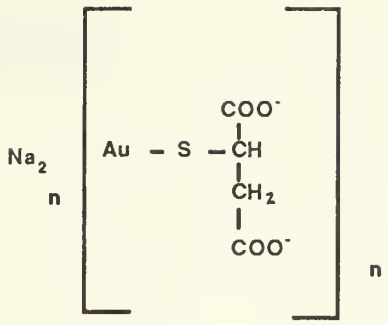
$$k_{obs} = k_3[I]/(K_I + [I]) \qquad (10)$$

Since this equation predicts hyperbolic saturation kinetics, a plot of $1/k_{obs}$ versus $1/[I]$ allows a graphical estimation of k_3 and K_I.

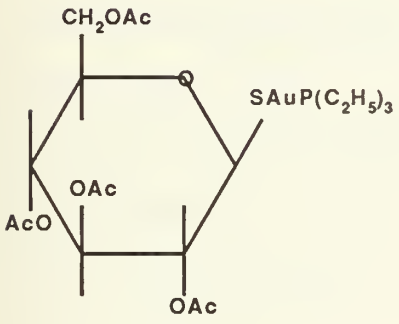
The purpose of this investigation was to isolate, purify, and partially characterize the enzyme hyaluronidase and then study the inhibition of the enzyme with a gold complex, Auranofin and compare it with a well known steroid, anti-inflammatory drug, 22-ketocholesterol oxime.



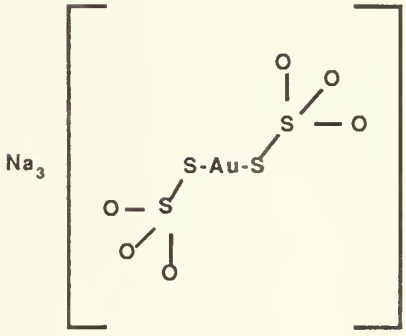
THIOGLUCOSOAURATE (I)
SOLGANOL



SODIUM THIOMALATOAURATE (I)
MYOCHRYSIN



S-2,3,4,6-TETRAACETYL- β -D
THIOGLUCOSO(TRIETHYLPHOSPHINE)
AURATE (I)
(AURANOFIN)



SODIUM BIS(THIOSULFATO-S)
AURATE (I)
(SANOCHRYSIN)

Figure 3. Gold Complexes Used in the Treatment of Rheumatoid Arthritis.

II. EXPERIMENTAL

Materials

The following materials were used.

Sigma Chemical Co.

Ammonium persulfate
Bovine serum albumin
p-Dimethylaminobenzaldehyde
Hyaluronic acid
Hyaluronidase
Potassium tetraborate
Sephadex G-75
N,N,N',N',-Tetramethylethylenediamine
Trizma 7-9

J.T. Baker Chemical Co.

Hydrochloric acid

Calbiochem-Behring Corp.

Hydroxylapatite

Florida Distiller Co

Absolute ethanol

Eastman Kodak Co.

Coomassie Blue R-250
N,N', methylene-bisacrylamide
Photo-flo 200 solution

Smith Kline & French Laboratories.
Auranofin

Dr. Edward Parish.
22-ketocholesterol oxime

Allied Chemical

Bromophenol Blue

Fisher Scientific Co.

Acetic acid
Acrylamide
Formic acid
Sodium acetate
Sodium chloride
Sodium hydroxide
Sodium phosphate monobasic
Trichloroacetic acid
Sucrose

Apparatus

1. Electrophoresis apparatus for slab gels	Hoefer Scientific Instr.
2. Mettler balance	Mettler Instrument Corp.
3. Uv/Vis Spectrophotometer Model no. 250	Gilford Instrument
4. Laboratory oven	Grieve-Hendry Co. Inc.
5. Fraction collector	Isco Inc.
6. Superspeed RC2-B Automatic refigerated centrifuge	Sorvall Co.
7. pH-meter	Radiometer-Corporation
8. Incubator	Precision Scientific Corp.
9. Hot-plate/Stirrer	Corning Glass Work
10. Pipetman	Rainin Instrument Co.
11. Chromatography Column	Glenco Scientific Inc.

Sample Preparation

Human synovial fluids were obtained from the Hughston Sports Medicine Hospital and the Medical Center, Doctor's Hospital in Columbus, Georgia and the Internal Clinical Laboratories in Atlanta, Georgia. Animals under investigation at the Small Animal Clinic, College of Veternary Medicine, Auburn University, Auburn, Alabama were the source of canine synovial fluid.

Stored, frozen synovial fluid samples were thawed and centrifuged at 9,000 x g at 4°C in a refigerated centrifuge to remove cellular debris. The supernatant was collected and stored at -20°C for further experimentation.

Hyaluronidase Activity

Hyaluronidase was assayed by a modification of the method of Reisseg, Strominger, and Leloir.[100] The substrate stock solution of hyaluronic acid was 1.25 mg/ml and it was prepared by dissolving 20 mg of the hyaluronic acid in 16 ml of distilled water. Two hundred fifty µl of the 1.25 mg/ml substrate solution was mixed with 250 µl of 0.2 M formate buffer at pH 4.0 and 25 µl of the enzyme solution. The prepared samples were then incubated at 37°C for twenty hours. One hundred µl of 0.8 M potassium tetraborate was added to each assay tube and heated in vigorously boiling water for three minutes and cooled in tap water. Three ml of dimethylaminobezaldehyde reagent were added and after

mixing, the tubes were heated for twenty minutes at 37°C. The tubes are then cooled, and the amount of N-acetylglucosamine groups released were measured at 585 nm. To calculate the concentration of the product N-acetylglucosamine, the gram molecular weight was taken to be 221.2.

Protein Concentration and Specific Activity

The protein concentration was determined using the Bradford protein assay. This method was based on the observation that the maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595 nm when binding to proteins.[101,102] The Bradford protein assay is commercially available as a kit from Biorad; containing lyophilized protein (bovine serum albumin) and a dye reagent provided as a five-fold concentrate. The dye was diluted, four parts distilled water to one part dye, and then filtered through Whatman number one filter paper. The standard assay contained 5-500 µg of protein. The absorbance was measured on the spectrophotometer at 595 nm at 25°C. Specific activity was defined as the µmol/min/mg of protein at 25°C.

Purification of Hyaluronidase

Human synovial fluid collected from different patients were pooled and stored for further use. The pooled portions were diluted 1:1 volumetrically with ice cold buffer

containing 0.1 M acetic acid, 20% sucrose, 1.0 mM EDTA and 0.01% Triton X-100 at pH 5.0. A Sorvall refrigerated centrifuge with a SS-34 rotor was used to centrifuge the homogenate at 12,000 x g for 40 minutes at 4°C. The supernatant solution was filtered through glass wool to remove any particles floating in solution and then adjusted to pH 5.0 by addition of 0.5 M acetic acid buffer. This solution was utilized for the first step in the purification. Batches of 50-70 ml each were used in the following procedure.

Sephadex G-75-120 Chromotography

The method descibed by Reiland [103] was used to prepare the Sephadex beads. Two liters of distilled water were used to suspend twenty-five grams of Sephadex G-75-120 powder, which was then heated in a water bath at 70°C for three hours and allowed to stand overnight. The supernatant solution was discarded and the remaining slurry was degassed and poured into a 2.5 X 110 cm glass column. A 0.01 M sodium acetate buffer containing 1.0 mM EDTA and 0.1 M NaCl at pH 5.0 and 4°C was used to equilibrate the packed column for 24 hours at which time a flow rate of 30 ml/hr was established. Seventy ml of the supernatant solution from the previous step was applied to the top of the column and the sample was eluted with the same equilibrating buffer at a flow rate of 25 ml/hr. The eluents were collected in 5.0

ml fractions and analyzed first for protein content in the spectrophotometer at 280 nm. The hyaluronidase activity for each protein fraction was measured as previously described. The enzyme fractions were pooled and used in the next purification step.

Hydroxylapatite Chromotography

The active enzyme fraction from the G-75 column was applied to a 1.5 X 4.5 cm column of hydroxylapatite (Calbiochem) which had been equilibrated with 10 mM sodium phosphate buffer containing 1 mM EDTA at pH 7.0 for six hours. The column was eluted with the same equilibrating buffer at a flow rate of 18 ml/hr and then eluted with 200 ml of a 10-250 mM sodium phosphate gradient. One and one-half ml fractions were collected at a flow rate of 18 ml/hr. The fractions were analyzed for protein content and hyaluronidase activity as previously descibed.

Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed on the samples using slab gels. Theoretically, electrophoresis through polyacrylamide gels should lead to enhanced resolution of a sample compound because the separation is based on both molecular sieving and electrophoretic mobility. Polyacrylamide gels were prepared by the free radical polymerization of acrylamide and the crosslinking agent, N-N'-methylene-bis-acrylamide. Chemical

polymerization is controlled by an initiator/catalyst system, ammonium persulfate, N,N,N',N'-tetramethylenediamine (TEMED).[104]

The gel stock solutions were prepared according to the method of Davis.[105] The solutions were stored at 4°C for two weeks while the ammonium persulfate was freshly prepared prior to use. Equilibration and degassing of the solutions were completed before polymerization with the catalyst. The glass plates were pretreated with Kodak photo-flo 200 solution prior to usage to enhance eveness of gels.

To perform the polyacrylamide gel electrophoresis the following reagents were prepared.

1. 10% separating gel:
   13.5 ml of a 22.6% acrylamide and a 0.6% N,N'-methylene bis acrylamide solution
   15 ml of 0.375 M Tris buffer at pH 8.7
   0.025 ml TEMED
2. 3.5% Stacking gel:
   34 ml of a 22.6% acrylamide and a 0.6% N,N'-methylene bis acrylamide solution
   34 ml distilled water
   7.5 ml of a .0125 M Tris buffer at pH 6.8
3. Catalyst:
   A 10% solution of ammonium persulfate was made by dissolving 0.1 g of ammonium persulfate in 1 ml of distilled water.

4. Fixing solution:

   10% trichloroacetic acid

5. Stain stock:

   Coomassie Blue G-250 solution

6. Sample buffer:

   0.08 M Tris at pH 6.8

   1 M sucrose

   0.01% bromophenol blue (tracking dye)

   0.02 M EDTA

7. Stain:

   0.05% solution of the stain stock

   7% acetic acid

   5% methanol

8. Destaining solution:

   7% acetic acid

   5% ethanol

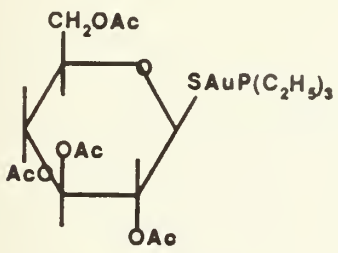
Procedure for Preparing the Gels

A 10% acrylamide gel was prepared as described earlier. The polymerization was initiated by the addition of the catalyst. After completion of the polymerization, the slab gel was layered with the stacking gel. The protein samples were diluted 1:1 with the sample buffer and electrophoresis was allowed to proceed for six hours at 8 ma/slab gel. The proteins were located using the Commassie Blue G-250 stain which was added to the gels with the protein samples. The

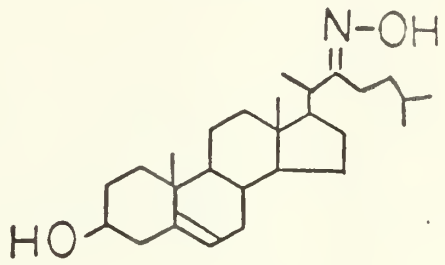
gels were placed in the fixing solution for fifteen minutes and then in the staining solution for overnight incubation. The stained gels were washed in the destaining solution for several hours with continuous shaking to destain the gel background. The finished gels were placed in plastic bags containing 7% acetic acid and stored at 37°C.

Inhibition studies

Hyaluronidase was tested for inhibition with a gold compound, Auranofin and a steroid, 22-ketocholesterol oxime.



(AURANOFIN)



22-KETOCHOLESTEROL OXIME

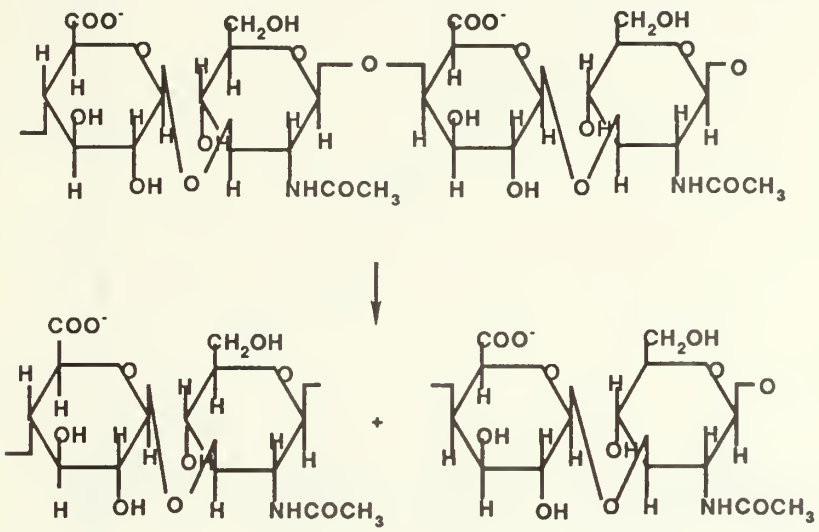
Hyaluronic acid was used as the substrate for the studies. Both the steroid and gold complex were first dissolved in absolute ethanol and further diluted in a 0.2 M phosphate buffer, pH 4.0 because of their insolubility in aqueous media. A constant concentration of enzyme (0.1 mg/ml) was mixed with different gold and steroid concentrations. The concentrations of Auranofin were varied from 1.0 mM to 10 mM. Fifty µl aliquots of gold complex-enzyme mixture were tested over a period of twenty-four hours for enzyme activity. The steroid concentrations were varied from

1.25 mM to 7.5 mM. The steroid enzyme-inhibitor mixtures were tested according to the procedure followed for Auranofin. Control experiments were conducted in the same buffer-ethanol mixtures. The control involved the use of the enzyme hyaluronidase without an inhibitory compound.

III. RESULTS

Enzyme Analysis of Synovial Fluid

The hyaluronate digestion catalyzed by the enzyme hyaluronidase is shown in the following equations.



(11)

As the enzyme catalyses the cleavage of the acetal bonds, N-acetylglucosamine end groups are formed and measured as described in the assay procedures. The activity was recorded as absorbance/hr and then converted to µmol/min of N-acetylglucosamine using the standard curve (Figure 4). The protein concentration was measured using the Bradford protein assay kit. The absorbance of protein was then converted to mg of protein by using the BSA standard curve shown in Figure 5. The specific activity of the enzyme was then determined in the units µmol/min/mg protein.

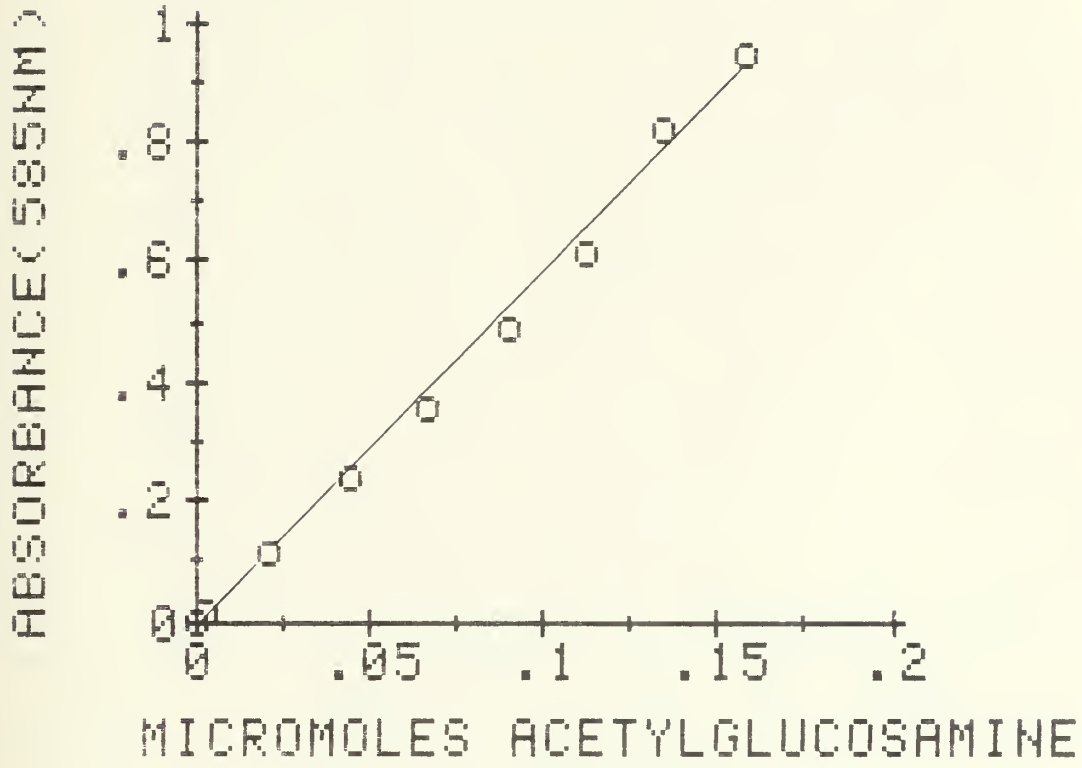


Figure 4. Standard Curve of Absorbance Versus Concentration of Acetylglucosamine.

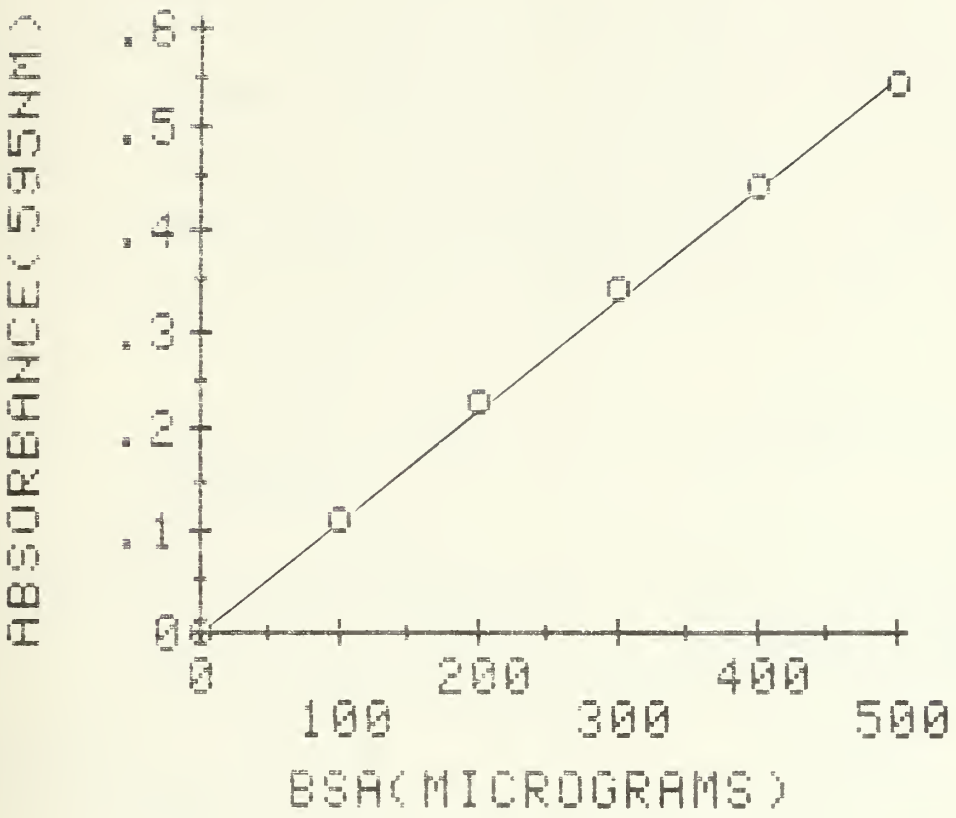


Figure 5. Standard Curve of Absorbance Versus Concentration of Bovine Serum Albumin.

Canine Synovial Fluid

The hyaluronidase activity in unpurified canine synovial fluid from 13 dogs was determined and the tabulated activities are shown in Table 3. A thirteen-fold increase was observed in the activity of the 4 rheumatic dogs as compared to the 7 normal dogs. In addition, 2 synovial samples tainted with blood contained approximately 40 fold greater hyaluronidase activity than normal synovial fluid and three times greater activity than fluid from rheumatic dogs.

Table 3. Comparison of Mean Hyaluronidase Activities in Canines

Condition	Number of Subjects	Mean X 10^3 Units/ ml synovial fluid
Rheumatoid	4	1.97±.24
Normal	7	0.15±.05
Blood tainted Synovial Fluid	2	6.02±.38

*Unit = μmole/min

Human Synovial Fluid

The purification of human synovial hyaluronidase by gel filtration thru Sephadex G-75-120 is illustrated in Figure 6. When measured at 280 nm, the elution profile revealed 3 protein peaks but only one peak contained activity. Peaks A & B showed no hyaluronidase activity while peak C (tubes 80-125) contained the hyaluronidase activity of

0.49×10^{-4} units/mg. One peak containing catalytic activity was obtained from the hydroxylapatite chromotography of the G-75-120 preparation. The elution profile is shown in Figure 7. The single peak yielded a specific activity of 1.1×10^{-4} units/mg which represents a four fold increase in specific activity. Table 4

Table 4. Purification Procedure for Human Synovial Fluid Hyaluronidase

Step	Activity (μmoles/min/ml)	Protein (mg/ml)	Specific Activity $\times 10^3$ (μmole/min/mg)
Homogenate (10,000 x g)	0.000866	32.1	0.028
G-75-120	0.00076	15.5	0.049
Hydroxylapatite	0.000376	3.4	0.11

Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed after purification to determine the purity of the enzyme. The protein bands were identified by staining the gels in a solution of Commassie Brilliant Blue, a dye which preferentially binds to proteins (Figure 8). From the polyacrylamide gel electrophoresis, a single band of the purified hyaluronidase was obtained (Figure 9). Using a series of proteins as molecular weight standards, a purified sample of hyaluronidase was estimated to have a molecular weight of 48,000.

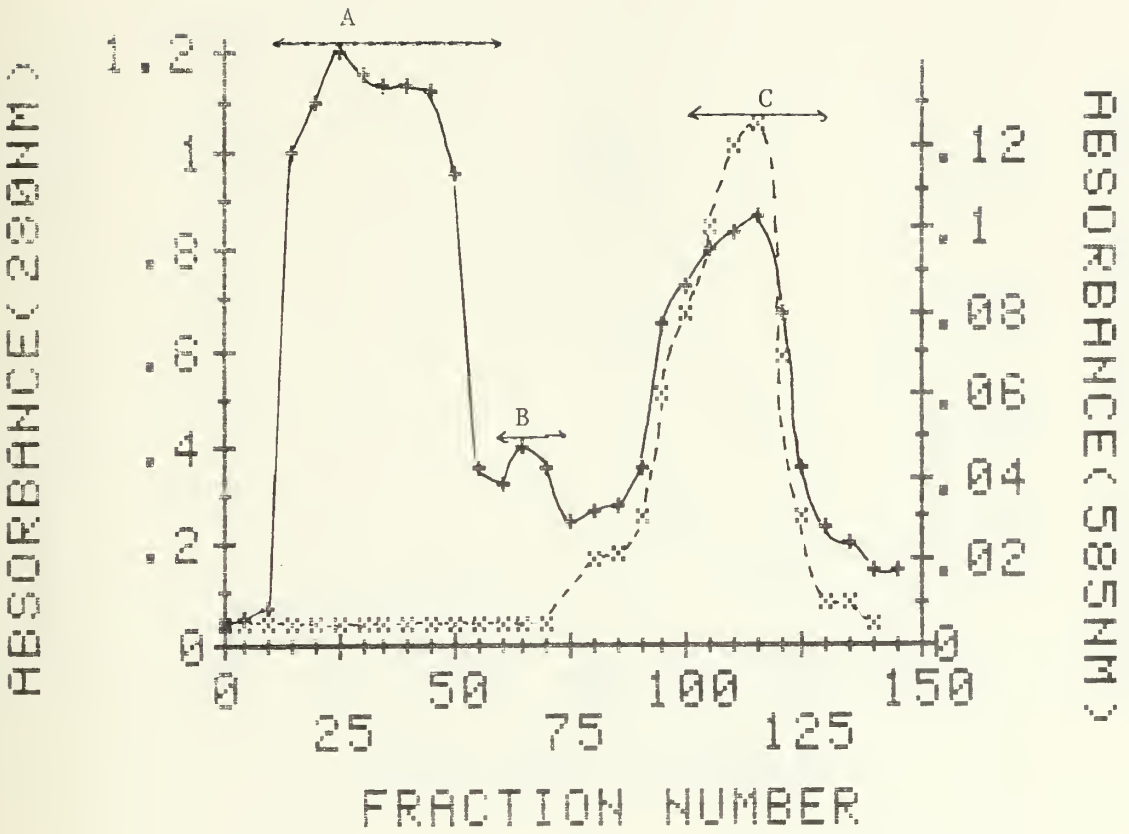


Figure 6. Chromotography of Human Synovial Fluid Hyaluronidase on Sephadex G-75-120 Column. The supernatant from step 1 was applied onto a 2.5 x 110 cm column and eluted with 0.01 M acetate buffer containing 0.01% triton X-100, 1.0 mM EDTA, at pH 5.0.
X----X, Enzyme activity; +——+, Protein profile

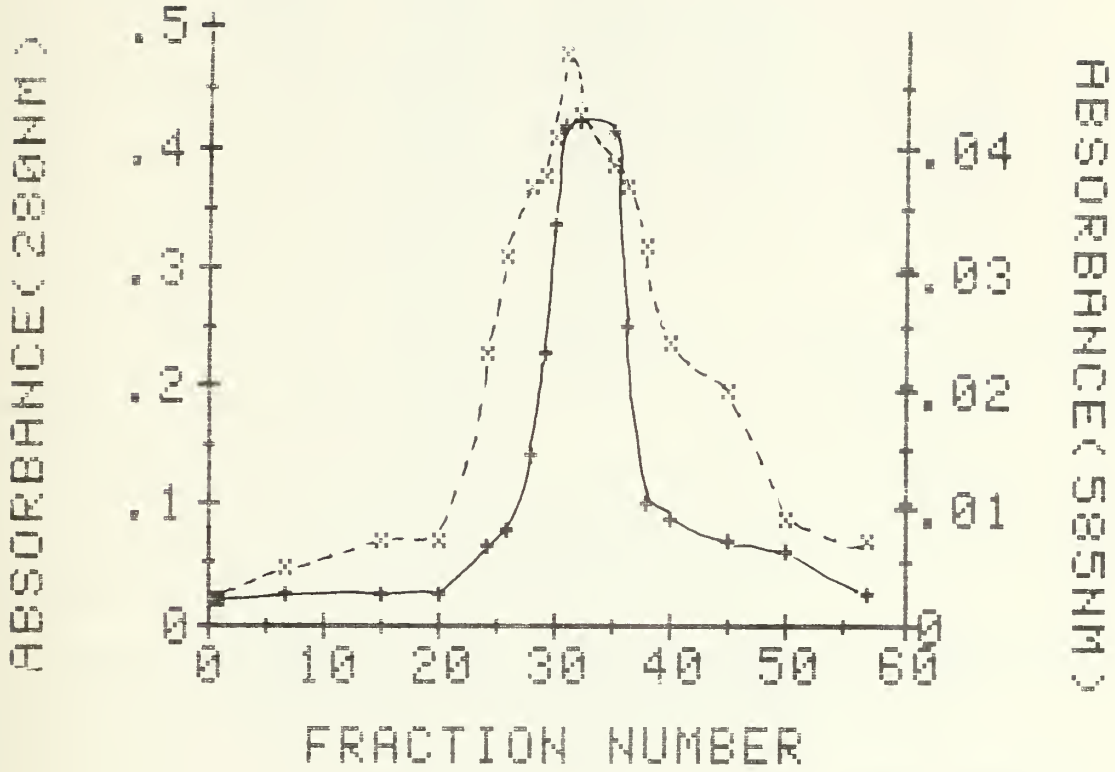


Figure 7. Chromotography of Human Synovial Fluid Hyaluronidase on Hydroxylapatite. The dialysate from step 2 was applied onto a 1.5 x 4.5 cm column, previously equilibrated with the dialysis buffer, 10 mM sodium phosphate, 1 mM EDTA at pH 7.0. The enzyme was eluted with 200 ml of a linear gradient (10-250 mM) sodium phosphate. X----X, Enzyme activity; +——+, Protein profile.

Enzyme Inhibition

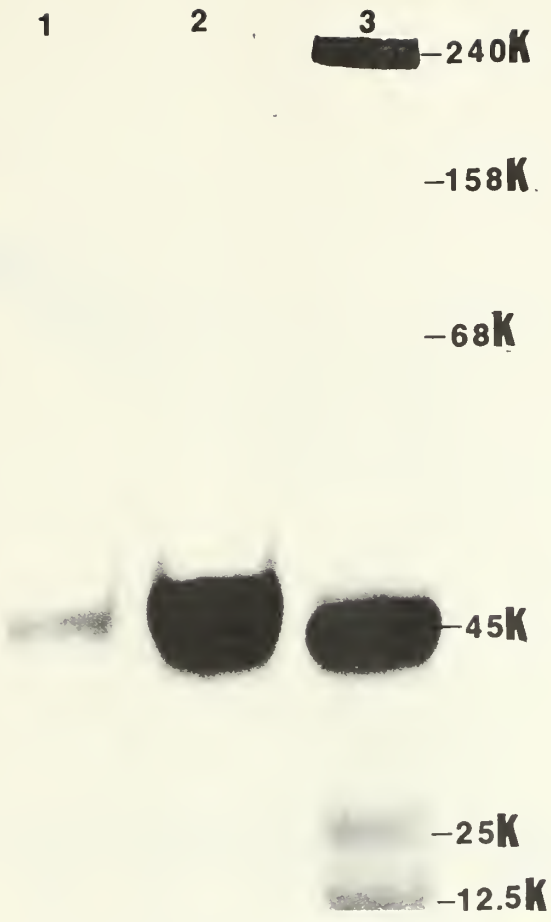
Hyaluronidase from human synovial fluid was inhibited by the gold complex Auranofin. The rate of inactivation is shown in Figures 10. A slow and gradual loss of enzyme activity was observed over a period of 24 hours. The control uninhibited hyaluronidase did lose 3% activity within 24 hours, however, the other curves have been corrected for the loss.

With the use of the semilog plot of remaining activity, a linear relationship was developed to an inactivation level of 45% as seen in Figures 10 & 11. Using the double reciprocal plot(Figure 12), Auranofin yielded a K_I of 8.78 mM and a k_3 of 0.049 min^{-1}.

The steroid derivative, 22-ketocholesterol oxime, was analyzed for inhibition and kinetics. The rate of inactivation is shown in Figure 13. It was observed that 22-ketocholesterol oxime induced an inhibition of enzyme activity which plateaued by six hours. A semi-log plot of data in Figure 13 did not yield straight lines indicating the absence of the irreversible step leading to formation of the covalently bonded E-X complex (Figure 14). A Lineweaver-Burk plot of the steroid induced inhibition yielded parallel straight lines thereby suggesting an uncompetitive inhibition behavior (Figure 15).

1. Osteoarthritis (Human)
2. Rheumatoid Arthritis (Human)
3. Rheumatoid Arthritis (Canine)
4. Normal (Canine)

Figure 8. Protein Staining of the Gel Electrophoresis for Human Versus Canine Synovial Fluid.



1. Hydroxylapatite Peak
2. Sephadex G-75-120 Peak C
3. Protein Standards

Figure 9. Protein Identification Bands of Hyaluronidase Purification with Protein Standards.

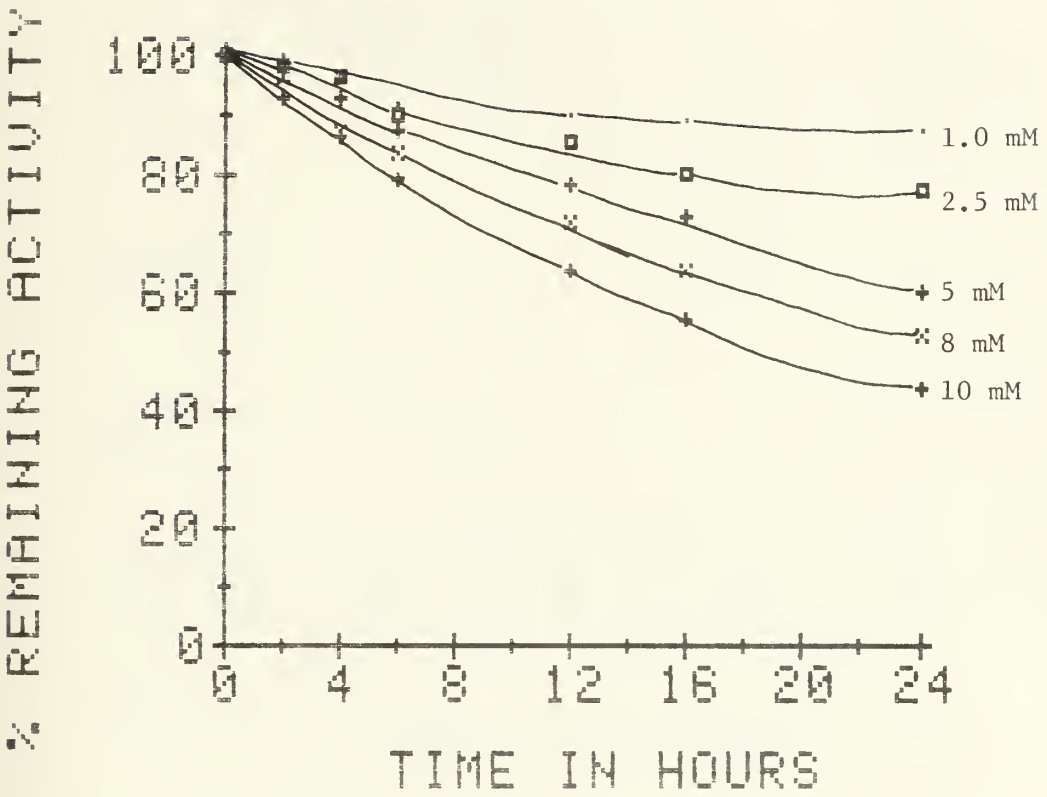


Figure 10. Time Course Inactivation of Hyaluronidase by Auranofin.

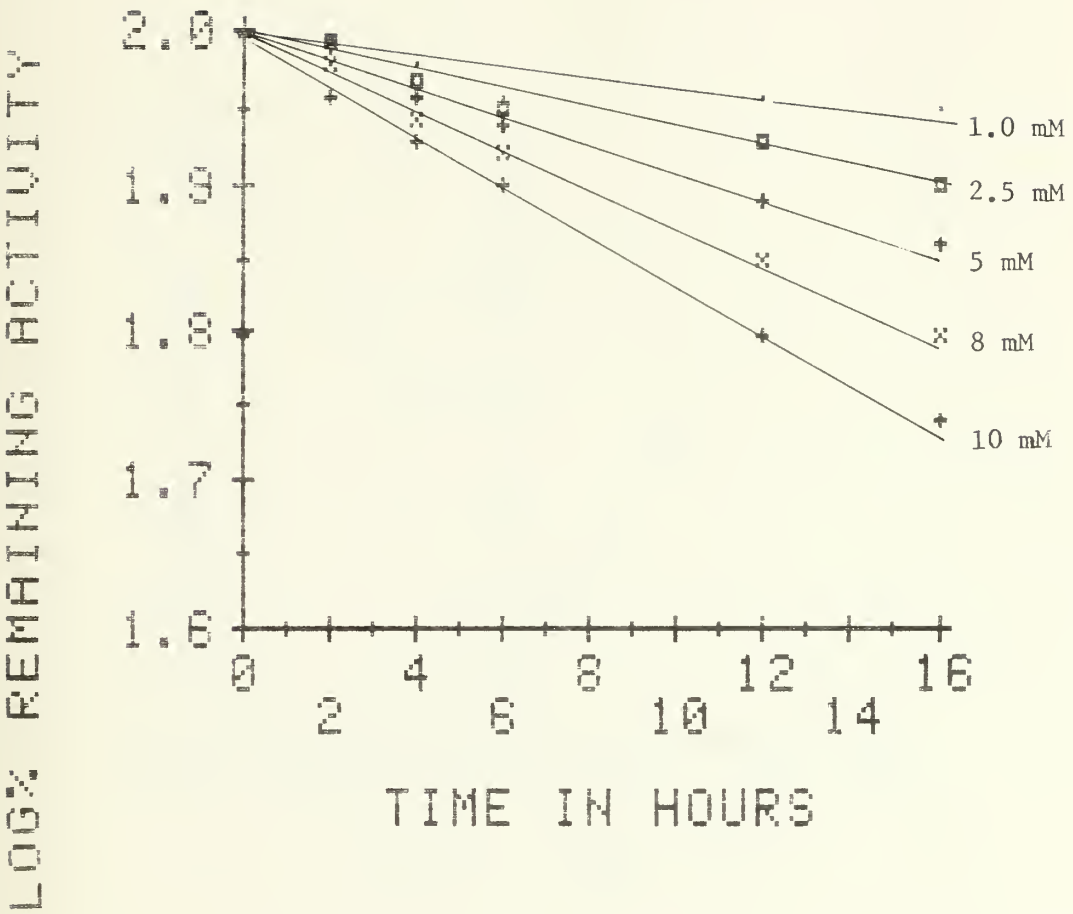


Figure 11. Semilog Plot of Data From Figure 10 Against Inactivation Time by Auranofin.

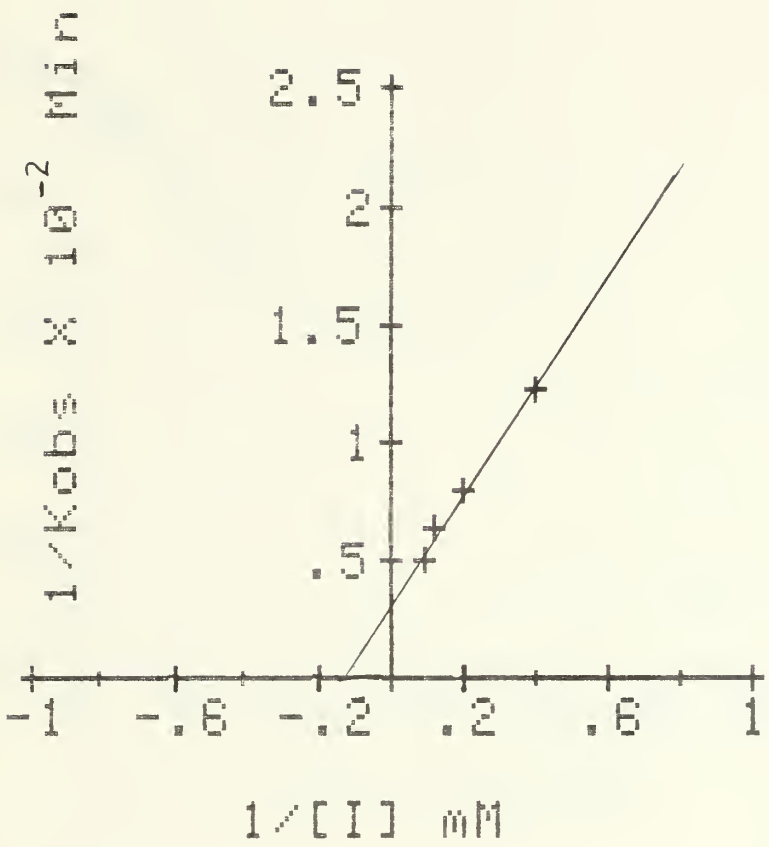


Figure 12. Reciprocal Plot of Pseudo First Order Rate Constant with the Auranofin Concentration.

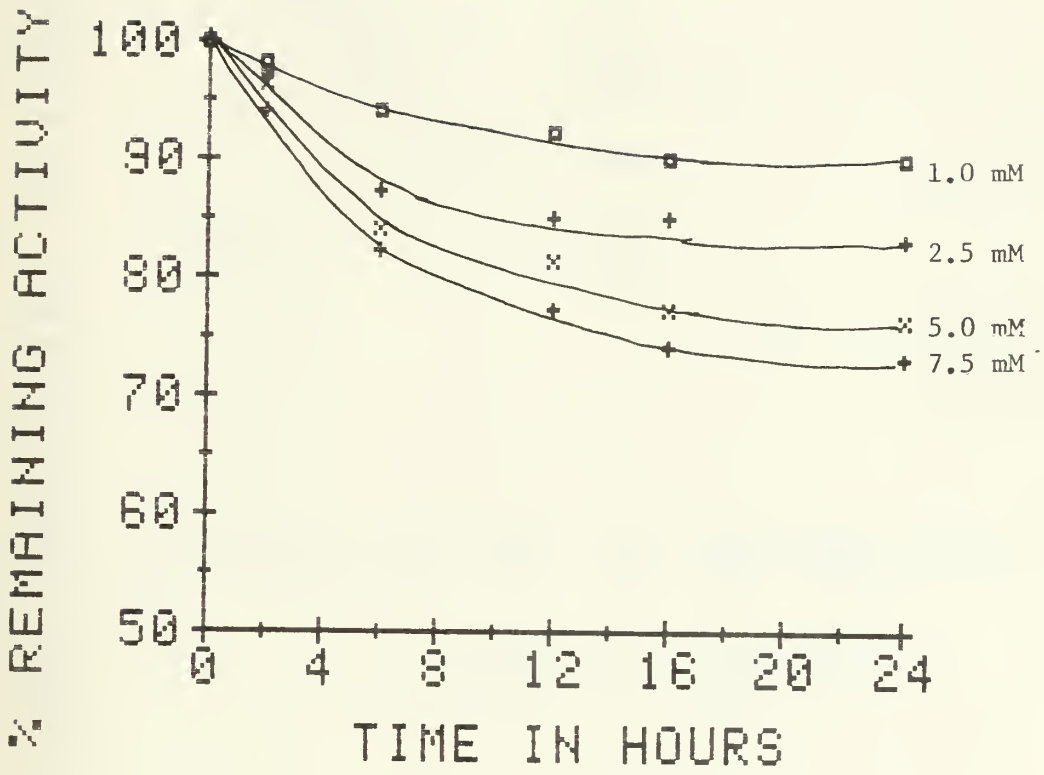


Figure 13. Time Course Inactivation of Hyaluronidase by 22-Ketocholesterol Oxime.

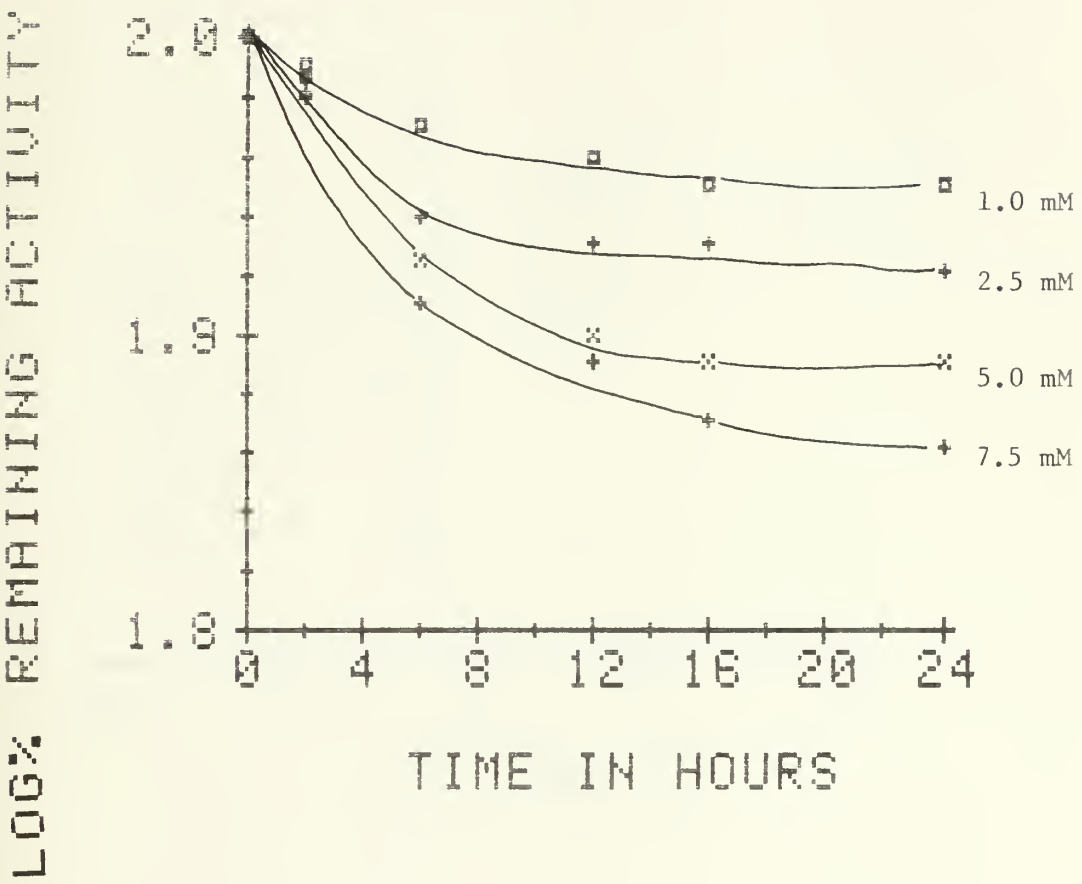


Figure 14. Semilog Plot of Data From Figure 13 Against Inactivation Time by 22-Ketocholesterol Oxime.

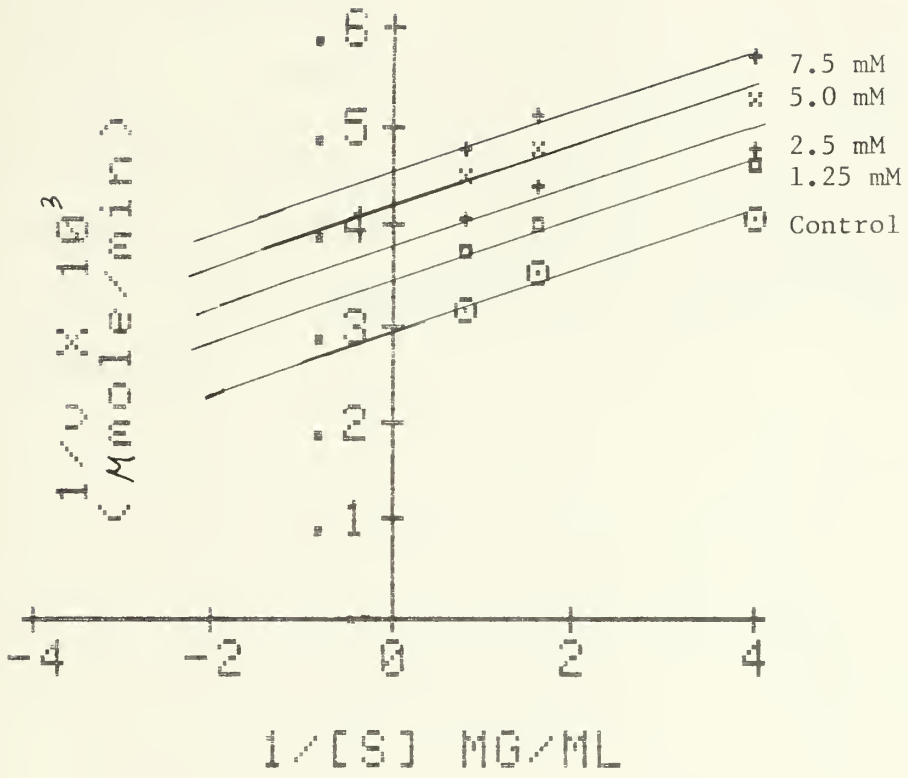


Figure 15. Lineweaver-Burk Plot of the Inhibition of Hyaluronidase by 22-Ketocholesterol Oxime.

Secondary plots of the reciprocals of $Vmax_i$ against the [I] concentrations gave straight line curves best fitted in the following equation.[106]

$$1/Vmax_i = 1/VmaxK_I[I] + 1/Vmax \qquad (12)$$

The kinetic constants for the inhibition of hyaluronidase by 22-ketocholesterol oxime evaluated directly from Figure 16 were 11.86 mM and 30.3 μmole/min for K_I and Vmax, respectfully.

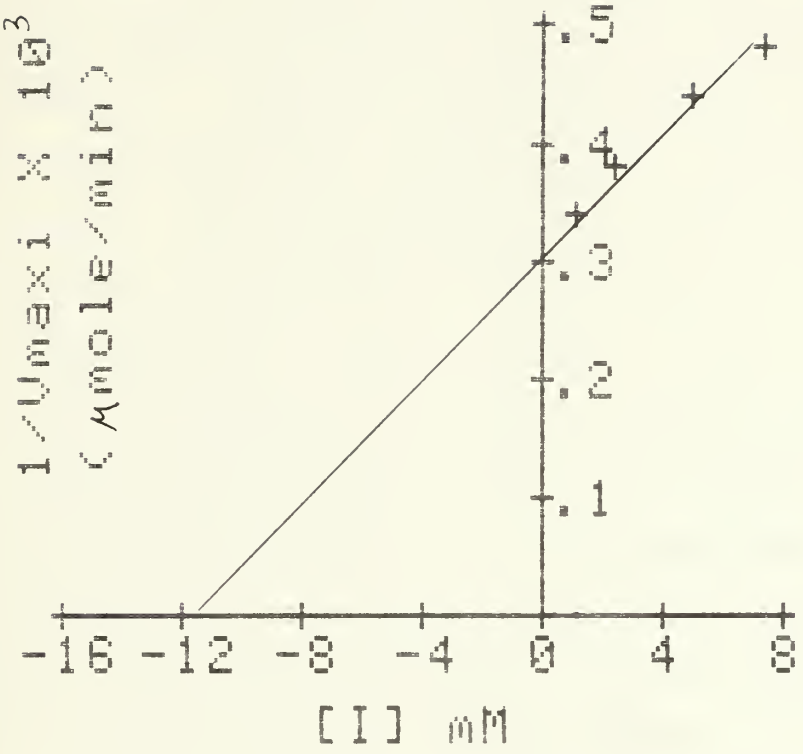


Figure 16. Secondary Plot Computed from the Lineweaver-Burk Curves in Figure 15.

IV. CONCLUSION

Hyaluronidase was purified from human synovial fluid following a modified procedure based on the work of Aronson and Davidson [67]. Using the modified procedure, human synovial fluid produced relatively smaller quantities of active enzyme. Upon completion of the purification, a specific activity of 1.1×10^{-4} µmole/min/mg was obtained which corresponds to a 4-fold increase in purification. Comparison of the specific activity of hyaluronidase from human synovial fluid characterized in the present study with hyaluronidase isolated from rat liver, honey bee venom, and pig liver is difficult due to the different assay procedures. The marked difference in assay conditions of Joy et al[35] (0.1 M sodium citrate buffer, pH 4.5, 0.15 M NaCl) for pig liver and of Kemeny et al[68] (0.1 M sodium acetate buffer, pH 6.0, 0.15 M NaCl) for honey bee venom versus those used in the present study (0.2 M sodium phosphate buffer, pH 4.0) preclude a direct comparison of enzyme activity.

The G-75-120 chromotography produced three separate peaks; the first two with no enzyme activity while the third peak contained the enzymatic activity. On elution through the hydroxylapatite column, one enzyme peak was produced which corresponds to previous studies. As reported by

Aronson and Davidson,[67] the purification procedure through the hydroxylapatite column allowed enhanced purification during each experiment but the loss of the enzyme varied from 30% to 75%.

The purified hyaluronidase was quite stable during the assay procedures using a 0.2 M formate buffer, pH 4.0. After the required twenty hour incubation time, the enzyme had no notable loss of activity. Polyacrylamide gel electrophoresis of the purified enzyme yielded only one major band as seen in Figure 8.

A separate study was conducted to compare the profile of hyaluronidase in human versus canine synovial fluid. The gel electrophoresis patterns, Figure 9, produced common protein bands from both human and canine synovial fluid samples. As seen in previous studies,[107] from our laboratory, it has been demonstrated that the acid phosphatase enzyme activity levels in human synovial fluid are higher in rheumatic synovial fluid as compared to normal fluid. The findings of the present study showed a thirteen fold increase in hyaluronidase activity from the synovial fluid of rheumatic dogs as compared to the non-rheumatic control dogs. (Table 3).

The levels of hyaluronidase in rheumatoid synovial fluids of humans do not significantly differ from those of osteoarthritic fluids.[74] It has been suggested that the enzyme is not produced locally in the joint by inflammatory

synovial cells.[74] In a comparative study, two samples of canine synovial fluid tainted with blood produced hyaluronidase levels three to forty times the amount seen in corresponding synovial samples(Table 3). With a determined molecular weight of 48,000 for human synovial fluid hyaluronidase as compared to 45,000 for serum albumin, it is possible that both canine and human synovial fluid hyaluronidase are derived by diffusion from the blood as discussed by Stephens,et al.[74]

After purification, the inhibition of hyaluronidase by a gold complex, Auranofin, and a steroid derivative, 22-ketocholesterol oxime, was investigated. Both compounds had inhibitory effects on the enzyme. Auranofin caused a steady decrease of hyaluronidase activity over the twenty-four hour period as seen in Figure 10. The initial rate constants were calculated from the reciprocal plot (Figure 12). As outlined in the previous section (eq.2), the evaluation of K_I is dependent on the formation of the reversible complex (E * I) which is in equilibrium with the free enzyme, and then reacts slowly to form the stable covalently bonded E - X complex. The rate constant k_3 represents the irreversible activation and this step represents the covalent bonding of the Au to a nucleophile such as S or possibly the N of a histidine, lysine or arginine. Several studies have shown that the above binding is seen in reactions of Pt(II)[108] as well as Au(III)[97]

complexes containing sulfhydryl groups at the active site. Our studies showed that Auranofin had a 10% to 55% inhibitory effect on hyaluronidase activity (Figure 10).

In this study, the steroid exhibited behavior suggestive of a reversible type of association with the enzyme hyaluronidase. Employing an inhibition equilibrium study, we were able to obtain results and calculate associated parameters. As discussed in previous publications from our lab, the mode of inhibition by 22-ketocholesterol oxime may be best explained by the location of the nitrogen atom in the aliphatic hydrocarbon chain of the steroid derivative since the rest of the molecule is unreactive. The actual role of the nitrogen atom has yet been determined.[107]

The results of the present studies show a definite difference between the inhibition by the gold complex versus the steroid derivative. Auranofin probably has an intermolecular interaction with the enzyme due to the formation of the covalent complex E - X rather than the weak association complex. This can be supported by the high K_I value of 8.78 mM and the relatively small rate constant of 0.05 min^{-1}. The small rate constant may represent the long time requirement exhibited by the gold complex to elicit the permanent inhibitory effects. The steroid derivative had a K_I of 11.86 mM and showed formation of a reversible equilibrium complex. As other studies have shown, steroids

elicit temporary relief in inflammation with the possiblity of forming an equilibrium complex.

The results obtained in the present study dealing with Auranofin and 22-ketocholesterol oxime are similar to those obtained by Haddad[107] in our laboratory. She evaluated the kinetics of Auranofin-induced inhibition of acid phosphatase obtained from human synovial fluid. She showed Auranofin inhibited acid phosphatase with a K_I of 0.545 mM and a k_3 of 22.7 min^{-1}. Since both studies show weak inhibitor-enzyme association as illustrated by similar K_I values and slow rates of covalent bond formation as illustrated by similar k3 values for Auranofin, it is suggestive that the interaction between the enzyme and gold complex is more likely to involve a weak nucleophile such as nitrogen rather than a sulfhydryl group or at least not a fully accessible sulfhydryl group as is expected for stronger gold-enzyme interactions. However, these observations need to be tested by further experimentation. When comparing the K_I's of the steroid derivative with the gold complex (as calculated from the Lineweaver-Burk plots Figure 12 & 16), the similarity in the values with the findings of Haddad[107] suggest a similar bonding profile. It is possible that both inhibitory complexes initially associate with the same groups on the enzyme or by a similar mechanism. However, the specific mechanisms of intermolecular interactions must be shown by further experimentation. After both inhibitors form the

initial equilibrium association for a period of time, the gold eventually forms a stable complex with a strong bond while the steroid is released. It is hard to assess what role the geometry of the molecules might have but may be reflected in the observed differences.

Therefore, it can be concluded that both the gold complex and the steroid derivative are inhibitors of hyaluronidase. With the use of a suitable test model and further experimentation, it may be possible to elucidate the molecular interactions of various anti-inflammatory drugs.

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